NOTE ON THE EFFECT OF X-RADIATION ON FERTILIZIN.[1]

A. RICHARDS AND A. E. WOODWARD.

The observations of one of the writers that x-rays would produce changes in the activity of certain enzymes suggested that these rays might perhaps be effective in bringing about changes in the action of cell extractives, particularly of fertilizin, the descriptions of which by Lillie and Glaser appeared at the beginning of the summer. The opportunity was presented to test this suggestion during the past summer at Woods Hole, since the one of us carried on studies on the effect of x-rays on some marine eggs and the other continued the work on fertilizin of *Arbacia* begun by Glaser. This note gives a summary of the results. It is realized by the writers that the study is by no means a complete one, but it is believed by them that publication is justified in view of the facts that the experiments give clear evidence on the main point under investigation and that there is at present no prospect of opportunity for their further work on the problem.

In taking up these experiments the writers felt that if it should be shown that x-radiation influences the activity of the cell extractive called fertilizin, that fact would be of interest from several view points: (1) without regard to the nature of fertilizin or its rôle in the fertilization of the egg, it is a substance derived from the eggs which has the property of being definitely modified by those external agents of which experimental use may be made; (2) in cell extractives, of which fertilizin is an example, there is a basis for the action of x-rays upon living cells, and doubtless the marked effects of the rays upon tissues is partially due to such action; (3) the modifiability of its activity by radiation is an interesting property of fertilizin; (4) this property may serve as a point in determining the relation of fertilizin to enzymes.

[1] Contribution from the Marine Biological Laboratory at Woods Hole, and from the Zoology departments of the University of Texas (No. 123) and of the University of Michigan.

The methods used in these experiments were largely those of Lillie and Glaser. The solution used as a standard was prepared according to the method of Lillie by "adding to a certain number of 'dry' eggs, double their volume of sea-water, and with occasional slight agitation allowing ten minutes to elapse. At the end of this time the ova were precipitated by 100 revolutions of the centrifuge and the supernatant fluid, a clear, golden liquid in the case of *Arbacia*," was decanted, (Glaser, 14*a*). The agglutination of fresh sperm in suspension by fertilizin in both control and radiated solutions, was tested by the unit concentration method of Lillie, of which he says, (13*b*) "The agglutination reaction of the sperm in the presence of this substance (*Arbacia* fertilizin) is, as noted in previous studies, reversible, and the intensity and duration of the reaction is a factor of the concentration of the substance. The entire reaction is so characteristic that it was possible to arrive at a unit by noting the dilution at which the least unmistakable reaction was given. This was fixed at about a five or six-second reaction, which is counted from the time that agglutination becomes visible under a magnification of about 40 diameters until its complete reversal. The unit is so chosen that a half dilution gives no agglutination of a fresh 1 per cent sperm suspension." Further details are given in his recent paper ('14, pp. 526–528). One can best observe the details of the reaction with the low power of the microscope. The sperm suspension is mounted under a cover glass and the drop of fertilizin added at the edge of the suspension by means of a pipette. The entire process is observed through the microscope, and the time elapsing before the complete reversal of the reaction is carefully noted by means of a stop-watch. Thus it was possible to determine the degree of activity of a given sample of fertilizin, and by comparing radiated and non-radiated solutions, to measure the effect of the radiation by x-rays.

Another possible method of studying the effect is suggested by the fact that fertilizin can be used to bring about the parthenogenetic development of *Arbacia* eggs, the so-called auto-parthenogenesis. The efficacy of fertilizin before and after radiation in bringing about auto-parthenogenesis is a measure of the action of the radiation on it.

In all the experiments with sperm it has been our policy to use only data from clear cut reactions in which the beginning and the end of the agglutination were definitely marked. Precautions were taken to see that the sperm suspension was fresh and clean. Lillie has shown that both of these factors are important, for an old suspension becomes inactive and the presence of impurities such as blood acts as an inhibitor of the reaction.

Previous experience (Richards, 14) has shown the radiations to be of three kinds in relation to their effect on enzymes depending on duration, intensity and distance of the object from the x-ray tube; namely, accelerative, non-effective, and inhibitive. Under the conditions which usually prevailed in these experiments, a short exposure, of about 2 minutes, is accelerative; an exposure of about five minutes is non-effective; and one of longer duration becomes inhibitive. In view of these facts, similar exposures of fertilizin were made and the resulting activity tested as already explained.

In a preliminary experiment on July 8 the following figures were obtained as the average of a number of readings of the time elapsing before the complete reversal of the agglutination reaction after short and long radiation of fertilizin. The fertilizin solution used was about 2 per cent. standard strength (in this early experiment the strength was not accurately determined, but it is not strictly necessary under the conditions of this test that it should be known exactly). For the control, non-radiated solution the average reaction time was 32 seconds; for the 2-minute radiation the average time was 33 seconds; and for the 15-minute minute radiation it was 23½ seconds. This solution was then diluted to one-half and these figures obtained: Control, 19 seconds; 2-min. radiation, 20 seconds; 15-min. radiation, 16 seconds. This experiment is incomplete and the differences lie nearly within the limits of variation, but they suggest definitely that the short radiation rendered the fertilizin slightly more active (that is, enabled it to hold the sperm in agglutination longer), and the long radiation caused it to be less active than the control. More decisive data would have been given had the dilutions been continued to unit concentration, a fact which led to the adoption of that method in subsequent experiments.

In another experiment (July 14) a 1/50 dilution (2 per cent. standard) of *Arbacia* fertilizin was used. It was separated into four parts, of which one (Sc) was kept as a control solution, one (S2) was radiated 2 minutes, one (S5) five minutes, and the last (S7) seven and a half minutes. The results of these solutions when tested for their agglutination time at successive dilutions to unit concentration are given in the following table. ½Sc means control solution diluted to one-half; ¼ Sc, diluted to one-fourth, etc. The difference between two successive reaction times is marked *d*. Unit concentration is indicated by the asterisk(*).

TABLE I.

Successive Dilutions.	Reaction Time.	Value of *d*.	Successive Dilutions.	Reaction Time.	Value of *d*.	Successive Dilutions.	Reaction Time.	Value of *d*.	Successive Dilutions.	Reaction Time.	Value of *d*.
Sc.	34 sec.		S2	37 sec.		S5	34 sec.		S7	29 sec.	
$\frac{1}{2}$ Sc.	22 sec.	12	$\frac{1}{2}$ S2	23 sec.	14	$\frac{1}{2}$ S5	27 sec.	7	$\frac{1}{2}$ S7	22 sec.	7
$\frac{1}{4}$ Sc.	17 sec.	5	$\frac{1}{4}$ S2	15 sec.	8	$\frac{1}{4}$ S5	19 sec.	8	$\frac{1}{4}$ S7	17 sec.	5
$\frac{1}{8}$ Sc.	10 sec.	7	$\frac{1}{8}$ S2	11 sec.	4	$\frac{1}{8}$ S5	12 sec.	7	$\frac{1}{8}$ S7	12 sec.	5
$\frac{1}{16}$ Sc.	4–5 sec.*	5	$\frac{1}{16}$ S2	7 sec.	4	$\frac{1}{16}$ S5	5 sec.*	7	$\frac{1}{16}$ S7	8 sec.*	4
			$\frac{1}{32}$ S2	4 sec.*	3				$\frac{1}{32}$ S7	0 sec.	

Inspection of this table shows that the activity of S2 was increased by the short radiation, for five dilutions were required to reduce it to unit concentration, whereas that state was reached in four dilutions in the other three solutions; also the full strength of this solution held the sperm in agglutination longer than did that of the control, 37 against 34 seconds. In other words, Sc was 800 units agglutinating strength, S2 was 1,600 units, S5 and S7 were each a little over 800 units, and much below 1,600 units strength. (Lillie, '14, p. 527.)

The number of dilutions required in S5 was the same as in Sc and the sperm were agglutinated the same time by both solutions. This is in line with the previous experience that a radiation of about five minutes' duration under the conditions of these experiments is non-effective. However, these figures give an additional fact of possible significance which has not been entirely confirmed by other experiments either on fertilizin or on enzymes such as pepsin. If *d* represents the differences between the number of seconds required for the reversal of the reaction by successive dilutions, its value in S5 is practically a constant, 7; but in Sc and

S2 it begins as a large number and decreases rapidly: in Sc its successive values are 12, 5, 7 and 5, while for S2 they are 14, 8, 4 and 4. In S7 the values of *d* are smaller and decrease more slowly, being 7, 5, 5 and 4. This suggests that the laws governing the agglutination reactions by the various solutions are of different character. But in as much as this interesting result has not been generally obtained it is not possible to attach special importance to it at this time. It is given merely as suggestive.

The data in the case of S7 indicate that the activity of the fertilizin was decreased although the number of dilutions was the same as in the control, because the number of seconds required for the reversal of the reaction at unit concentration was much larger than is usual; yet at a further dilution no reaction was obtained. Also the undiluted solution did not hold the sperm in agglutination as long as in the control. Furthermore, it may be significant that the value of *d* for S7, as indicated above, are smaller than in the case of the other solutions.

Subsequent experiments along the same line gave similar results. They show clearly that radiation by x-rays is capable of changing the activity of fertilizin, and in general agree with previous work that weak radiation is accelerative and strong inhibitive. Some of our experiments were performed during the latter part of the summer at the end of the breeding season and there were irregularities in the results, but it is believed that these irregularities may be attributed to the unsatisfactory condition of both sperm and eggs at this season of the year and that the statement above gives the true effect of radiation on fertilizin.

Also during the latter part of the summer the writers tested the effect of x-radiation on fertilizin with regard to its power of inducing auto-parthenogenesis. Due to the near end of the breeding season these results are not entirely trustworthy, but they agree fully on one point, namely, that the radiation effects changes in the capacity of fertilizin to induce parthenogenesis.

On August 10 a sperm agglutination experiment was performed which possibly throws some light on the irregularity of the auto-parthenogenesis and at the same time makes the auto-parthenogenesis test doubtfully applicable for the radiation problem. This experiment gave data showing that the radiation effects

wore off when the fertilizin had stood for some time. If this is true in general it must follow that, since the fertilizin must stand in the parthenogenesis experiments, there would be irregularity in the results.

The only tests of the effect of x-radiation on *Asterias* fertilizin were made on July 28, when the fertilizin was divided into four portions, as usual. One was kept for a control, one radiated two minutes, one five minutes, and the fourth fifteen minutes. The fertilizin was then put on mature *Asterias* eggs, which were allowed to stand two hours in the solution. They were then rinsed with sea-water and treated with hypertonic sea-water (50 c.c. sea water + 8 c.c. 2.5 M. NaCl) for thirty minutes, washed again with sea water, and allowed to stand for 12 hours. All four lots of eggs showed parthenogenetic development, and those treated with fertilizin which had been radiated 2 minutes had a much larger percentage of cleavages than either the control or the others.

Several times *Arbacia* fertilizin was similarly subjected to x-rays and then tested for its auto-parthenogenetic effect on fresh *Arbacia* eggs. The experiments are not satisfactory, because in most cases eggs from the same females gave abnormal results when tested in other ways. The following summarizes the more interesting experiments. Percentages were obtained by counting about 200 eggs.

TABLE II.

	Experiment I.		Experiment II.		Experiment III.		Experiment IV.		Experiment V.	
	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.
Sperm control			23.8		46.6					
Fertilizin control (unradiated)	30.2	0	9.5	0	5.2	1	20.2	.5	15.5	
Fertilizin 2 min. radiation.....	24.3	few	14.1	1	10.8	3	13.5	0	28.5	2
Fertilizin 5 min. radiation	21.6	"	17.9	0	8.4	0	10.5	0	87.2	
Fertilizin 15 min. radiation ...	17.5	0	15.6	.5	9.5	0	13.6	0	52.1	

Since the effect of x-radiation on fertilizin seems to be similar to its effect on enzymes, it is of interest to note the fact that the

efficiency of the agglutinin contained in fertilizin, like pepsin (Euler, p. 132) varies with the square root of the concentration. If the efficiency is measured by the number of seconds the sperm remain agglutinated, and the concentration is measured by units of strength, the curves in Figs. 1, 2, and 3 are obtained for the readings of July 14, August 10, and August 11, respectively. The average is shown in the dotted line of Fig. 4. If an equation is worked out for this curve, we obtain $y^2 = 11x$ where y represents the efficiency and x the concentration. This equation is plotted as a solid line on Fig. 4. In the higher dilutions, of which a greater number of values were averaged, and where readings could be made more accurately, the curves coincide very closely. In the less dilute portion the coincidence is not so marked, but is still within the limits of experimental error.

The writers are not now able to offer an opinion as to whether or not fertilizin has the character of an enzyme. The coincidence, however, in the behavior of this substance, when treated by x-rays, to that of true enzymes, is indeed striking.

While the nature and composition of fertilizin are as yet unknown, it is a cell-extractive which is capable of undergoing changes under the action of experimental agents such as radiation by x-rays. Possibly it, or its forerunner, exists in the egg in combination. Among the other constituents of *Arbacia* eggs, this substance stands as one which, at least in solution in sea water, is able to bring about certain reactions on the part of sperm, and these reactions are subject to experimental modification. This justifies the inference that this substance or perhaps some similar one within the egg may be capable of undergoing modification in its relations to the various intra-cellular activities.

In this modification we may look for the seat of part of the changes which are brought about in living tissues and especially egg cells by radiation. The Hertwigs, Packard and others have shown that the chromatin of such cells is affected, and there is good evidence that the cytoplasm as well is influenced. Changes in their activity have also been demonstrated in the case of enzymes. These experiments add still another to the list of substances which are affected by the action of x-rays. It is

probable that fertilizin is simply one example of a group of substances which may be the object of such action (but an example which may be studied). It is to be noted that these experiments render untenable the conclusion of the Hertwigs, that chromatin is the chief and perhaps exclusive seat of the effects of radiation upon eggs. Fertilizin is a substance doubtless without morphological representation in the structure of the egg; yet it may suffer considerable modification from x-ray treatment.

REFERENCES.

Euler, H.

'12 General Chemistry of the Enzymes. New York. Wiley.

Glaser, Otto

'13 On Inducing Development in the Sea Urchin (Arbacia punctulata) together with Considerations on the Initiatory Effect of Fertilization. Science, N. S., 38, p. 446.

'14a A Qualitative Analysis of the Egg-Secretions and Extracts of Arbacia and Asterias. BIOL. BULL., XXVI.

'14b On Auto-Parthenogenesis in Arbacia and Asterias. BIOL. BULL., XXVI.

Lillie, F. R.

'13a Studies of Fertilization. V. The Behavior of Spermatozoa of Nereis and Arbacia with Special Reference to Egg Extractives. Jour. Exp. Zool., 14.

'13b The Mechanism of Fertilization. Science, N. S., 38, p. 524.

'14 Studies of Fertilization. VI. The Mechanism of Fertilization in Arbacia. Jour. Exp. Zool., 16.

Richards, A.

'14 The Effect of X-Rays on Certain Enzymes. Amer. Jour. Physiol., 35.

FIGURE 1 – (JULY 14)

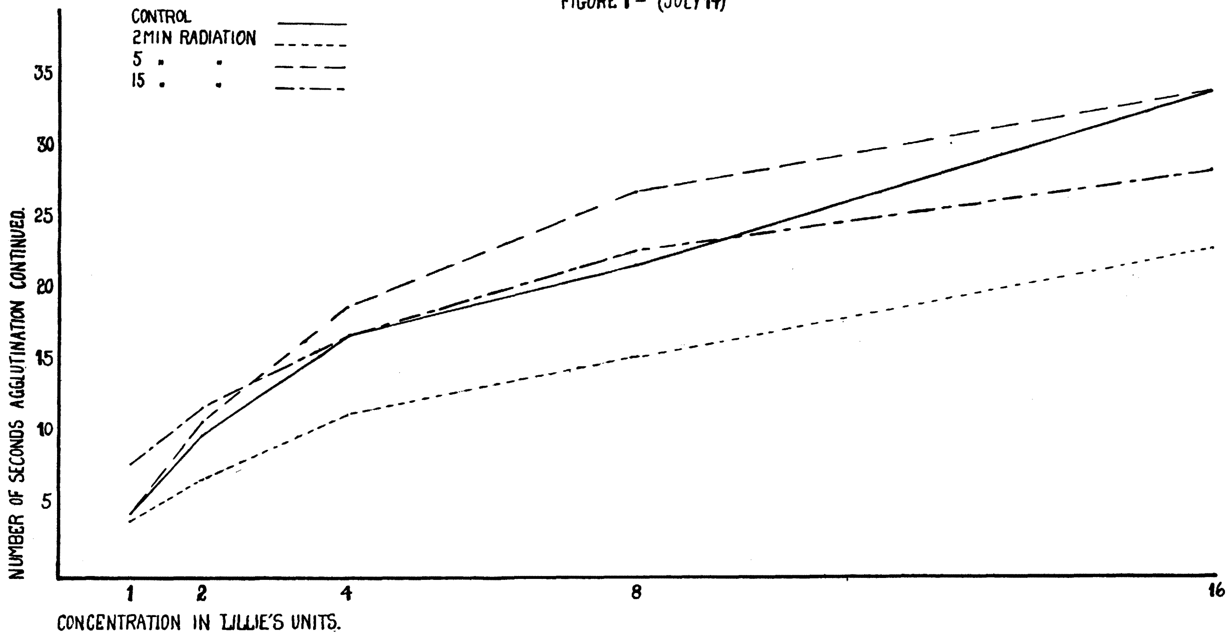
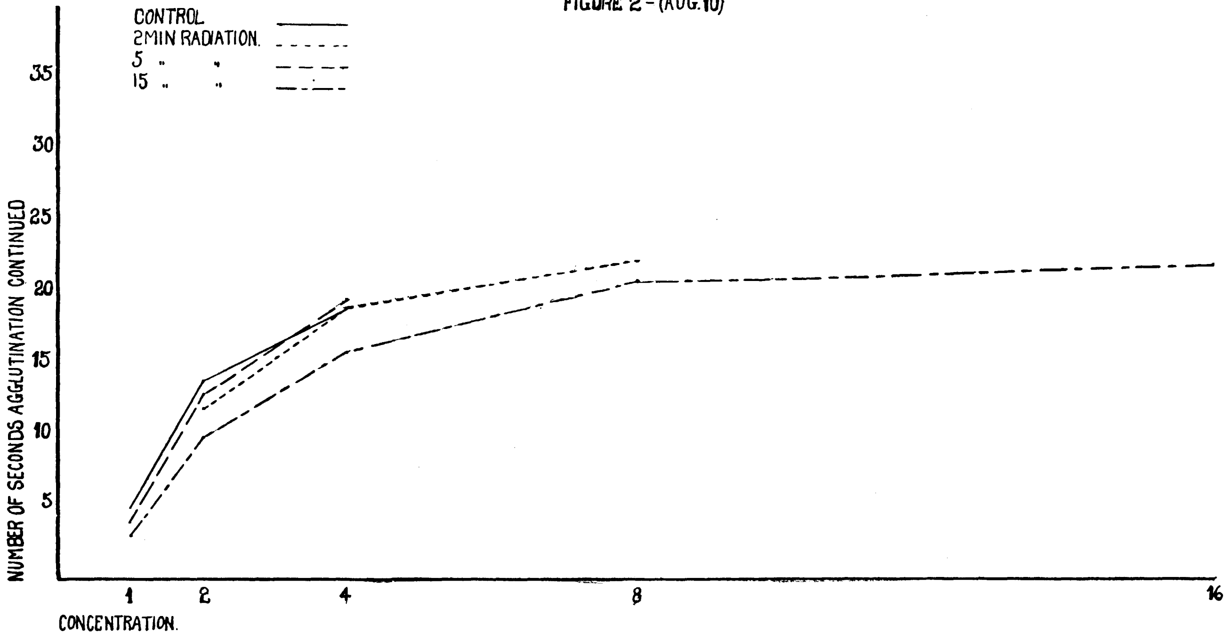
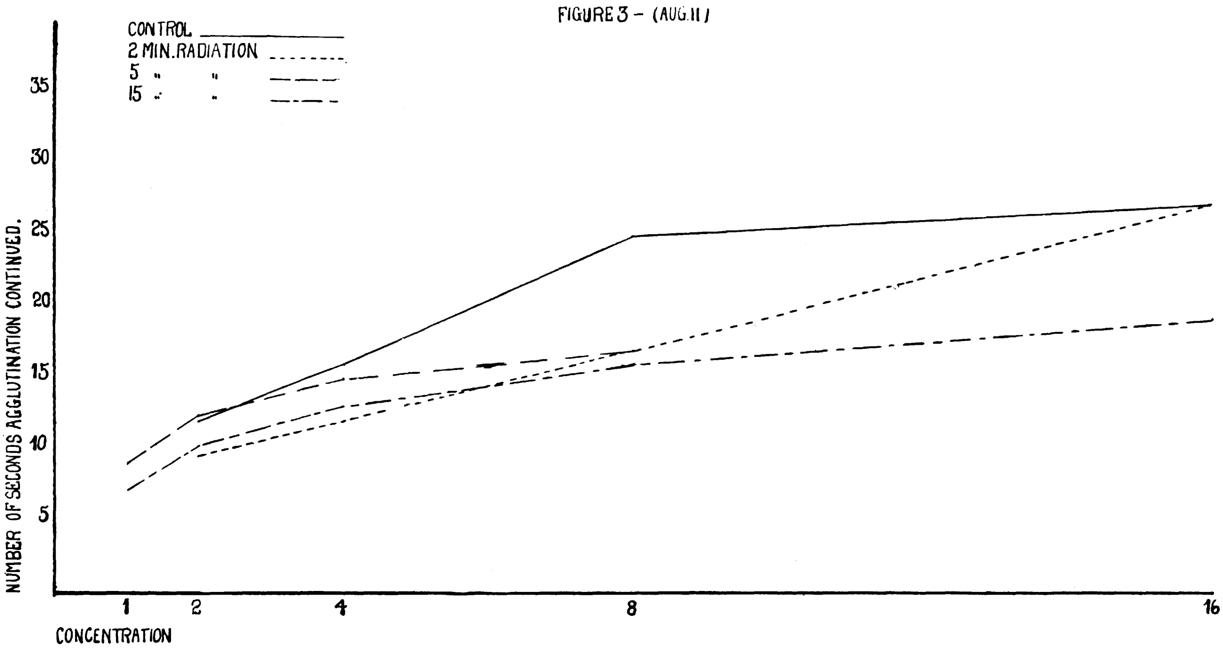


FIGURE 2 - (AUG. 10)

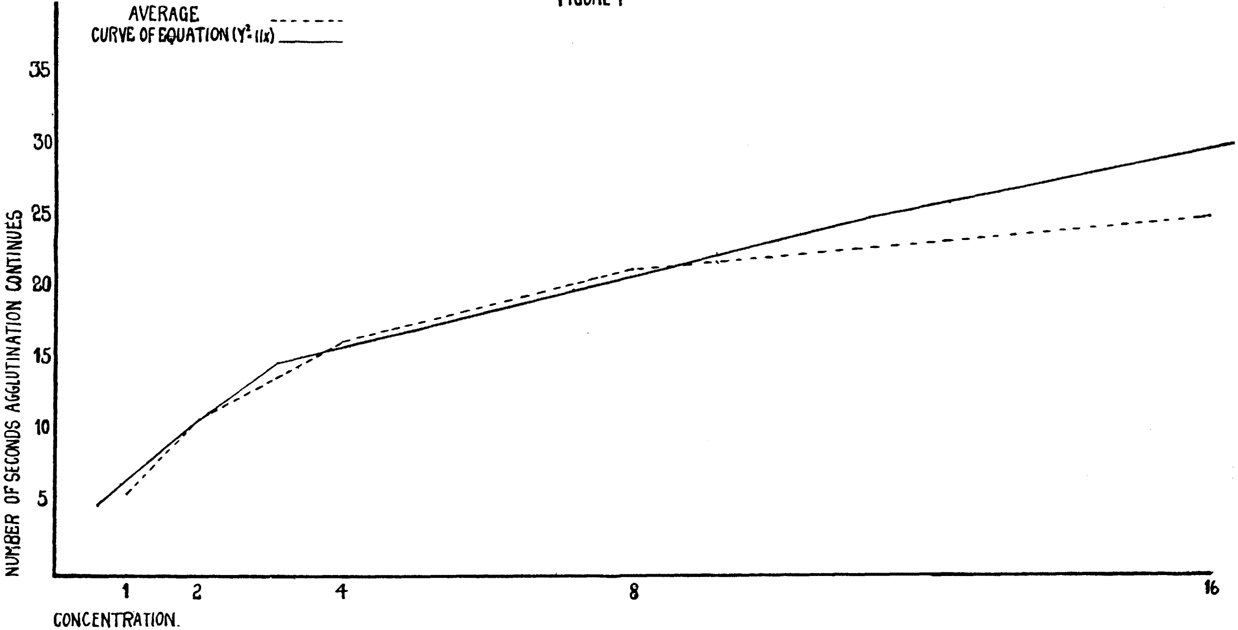


RICHARDS AND WOODWARD



RICHARDS AND WOODWARD

FIGURE 4—



RICHARDS AND WOODWARD